

CONTEMPORARY APPROACHES IN THE DIAGNOSIS AND STUDY OF *MYCOBACTERIA*: THE ROLE OF MICROSCOPY AND ARTIFICIAL INTELLIGENCE

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ABSTRACT

The genus *Mycobacterium* comprises structurally a complex of highly adaptive bacteria of major clinical importance. While tuberculosis remains a leading cause of mortality from infectious diseases worldwide, nontuberculous mycobacteria (NTM) are gaining increasing clinical relevance, particularly among immunocompromised patients. The unique structure of mycobacterial cell wall and the slow growth of these organisms necessitate the use of specialized microscopic approaches for their investigation and diagnosis.

Despite the advances in molecular diagnostics, microscopy remains a rapid and cost-effective key diagnostic tool, however limited by subjectivity and reduced sensitivity at low bacterial loads. The recent integration of artificial intelligence has significantly enhanced light and fluorescence microscopy by enabling an automated and standardized detection of the acid-fast bacilli.

The advanced high-resolution techniques, including electron and cryo-electron microscopy, provide detailed insights into mycobacterial ultrastructure and intracellular behaviour, contributing to a deeper un-

derstanding of their pathogenicity and drug resistance. This review summarizes the classical and the modern microscopic approaches and highlights their complementary roles in diagnostic and fundamental studies of mycobacteria.

Keywords: Nontuberculous Mycobacteria, microscopy, diagnostics

INTRODUCTION

The genus *Mycobacteria* includes structurally complex and biologically adaptive bacteria. They have a unique evolutionary strategy for survival in diverse ecological niches as well as within the human host [1–3]. The *Mycobacterium tuberculosis* complex (MTBC) remains a leading cause of mortality from infectious diseases worldwide, while nontuberculous mycobacteria (NTM) are increasingly recognized as important opportunistic pathogens, particularly in immunocompromised patients [4–6].

The unique structure of the mycobacterial cell wall, composed of peptidoglycan, arabinogalactan, and a dense layer of mycolic acids, underlies acid-fastness and limited permeability to dyes and drugs [1,2,7–9]. These characteristics necessitate the use of specific microscopic approaches for investigation. An additional challenge is the slow growth rate and prolonged generation time of mycobacteria [10–12]. Smear microscopy (SM) remains a key component of the diagnostic algorithm for tuberculosis and NTM infections, although it is typically used alongside other laboratory methods [6].

Despite the advances in molecular diagnostics, microscopy continues to play a major role in the early diagnosis of mycobacterial infections. Ziehl–Neelsen staining and its variants remain the standard technique for detecting acid-fast bacilli (AFB) using light or fluorescence microscopy [5]. Despite its high specificity (>98%), the method is semiquantitative and has limited sensitivity in paucibacillary samples [5]. Recent developments in artificial intelligence (AI) have introduced novel opportunities for automated analysis of microscopic images. AI-based algorithms enable standardized and high-throughput detection of AFB, improving the sensitivity, reproducibility, and workflow efficiency. Such technologies are particularly promising in resource-limited regions with a high tuberculosis burden. For more in-depth studies, of mycobacterial morphology, cellular organization, and cell dynamics, high resolution microscopic tech-

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niques such as confocal laser scanning microscopy and electron microscopy are used [10,12].

Transmission electron microscopy enables a detailed visualization of mycobacterial ultrastructure, including the multilayered cell envelope. It provides evidence for the intracellular interactions between mycobacteria and host macrophages, allowing assessment of phagosomal membranes and defects in phagosome maturation [13].

Recent advances in cryo-electron microscopy have further expanded these capabilities by enabling the structural analysis of mycobacterial components in a near-native state without extensive chemical fixation. This approach facilitates high-resolution investigation of molecular complexes involved in cell wall organization, metabolic pathways, and mechanisms of drug resistance [13].

Traditional Diagnostic Methods:

Light and fluorescence microscopy: classical approaches and their role in mycobacterial diagnostics

Light microscopy remains a cornerstone in the diagnosis and investigation of mycobacteria [5,14]. It allows the detection of AFB using the classical Ziehl-Neelsen staining method or its variants, such as the cold Kinyoun method. These techniques are based on the irreversible binding of carbol fuchsin to mycobacterial mycolic acids, which confers acid-fastness [6]. Historically, using specialized staining techniques Robert Koch identified *M. tuberculosis* and laid the foundation for modern mycobacterial diagnostics. The Ziehl-Neelsen staining, subsequently refined through the cumulative discoveries of Paul Ehrlich, Franz Ziehl, and Friedrich Neelsen, has become the most widely accepted standard for detection of AFB [5,14]. The main advantages of light microscopy include rapid turnaround time, low cost, and ease of application even in resource-limited settings, while providing direct visual evidence of AFB in clinical specimens. It uses heat to facilitate the penetration of the primary stain into the lipid-rich, waxy cell wall of mycobacteria, therefore designated as a „hot staining” method [1,2,7]. Although this barrier limits dye penetration, once the carbol fuchsin enters the cell it is retained during acid-alcohol decolorization, allowing the clear visualization of mycobacteria as red rods against a contrasting background. Beyond detection, Ziehl-Neelsen staining provides valuable morphological information, including the characteris-

tic cord formation by virulent *M. tuberculosis* strains, which is associated with pathogenicity [3,4]. In addition, microscopic examination allows the assessment of cellular integrity and approximate bacterial burden, contributing to treatment monitoring [5].

Despite its established role, light microscopy has important limitations. Its sensitivity is relatively low, requiring bacterial concentrations of at least 10^4 - 10^5 cells/ml for reliable detection [14]. As a result, early-stage and extrapulmonary tuberculosis may be missed. Furthermore, the method cannot distinguish between members of the *M. tuberculosis* complex and NTM, viable and nonviable organisms, or drug-susceptible and drug-resistant strains, necessitating complementary culture-based or molecular approaches [12].

Fluorescence microscopy constitutes an important extension of conventional light microscopy in mycobacterial diagnostics and research. The method relies on fluorochrome stains, including Auramine O and Auramine-Rhodamine, which bind to mycolic acid-rich components of the mycobacterial cell wall and emit bright yellow-green fluorescence when excited by UV or LED light [15,16]. This substantially enhances contrast and enables the rapid screening of large smear areas, resulting in a significantly higher sensitivity as compared to Ziehl-Neelsen staining [15,16]. In addition to improved detection, fluorescence microscopy provides valuable morphological information, allowing the visualization of subtle cellular alterations, granular forms, and aggregated structures that are not readily detected by standard light microscopy [9]. Such features are particularly relevant for nontuberculous mycobacteria, which exhibit greater morphological heterogeneity [4]. However, similar to acid-fast staining, this method does not permit species-level identification.

Recent advances in LED-based fluorescence microscopy have improved safety, reduced operational costs, and expanded applicability, particularly in laboratories with limited infrastructure [16]. A comparison of techniques for microscopy detection of genus *Mycobacterium* is presented in **Table 1**.

Ultrastructural look at the mycobacterial cell

Electron microscopy

Electron microscopy (EM) provides a resolution far beyond light-based methods, revealing structural features essential for the understanding of mycobac-

Table 1. Comparison of techniques for microscopy detection of genus *Mycobacterium*.

| Method | Principle | Stain | Sensitivity* | Advantages | Limitations | Main application | Selected references |
|--------------------------------|--|---|--|--|---|---------------------------------------|---------------------|
| ZN microscopy | Acid-fast staining with heat | Carbol fuchsin | Moderate (10 ⁴ –10 ⁵ bacilli/ml) | Low cost, easy, fast, standardized | Low sensitivity in paucibacillary samples; creating phenol aerosols | Routine diagnosis | 5, 14, 18, 19 |
| Kinyoun microscopy | Acid-fast staining without heat | Carbol fuchsin (high concentration) | Moderate (10 ⁴ –10 ⁵ bacilli/ml) | Simple and safer than ZN | Slightly reduced sensitivity vs. ZN | Routine diagnosis | 14, 19, 24 |
| Auramine–rhodamine microscopy | Fluoro-chrome binding to mycolic acids | Auramine O / Rhodamine | High (10 ³ bacilli/ml) 10% more sensitive than ZN | Increased sensitivity; rapid screening | Requires fluorescence microscope, potential toxicity of reagents | High volume laboratories | 14, 16, 19 |
| LED fluorescence microscopy | Fluorescence with LED illumination | Auramine O | High (with 5% more than Auramine–rhodamine microscopy) | Energy-efficient; durable | Higher initial cost | High volume laboratories | 14, 16 |
| AI-assisted microscopy | Automated image analysis | ZN / Fluoro-chrome | High | Speed, accuracy, is high efficiency | Requires validated algorithms; technical support | Screening | 15,16, 23,24 |
| Cryo-EM | Rapid freezing | Does not use conventional, heavy-metal stains | Very high | High resolution; 3D imaging | Expensive, researcher team | Advanced research | 17 |
| Electron microscopy (TEM/ SEM) | Electron beam imaging | Heavy metals | Very high | Ultrastructural detail | High cost, researcher team | Advanced research; structural studies | 1, 2, 3, 10, 17, 20 |

Abbreviations: ZN - Ziehl–Neelsen; LED - light-emitting diode; AI - artificial intelligence; TEM - transmission electron microscopy; SEM - scanning electron microscopy.

*Sensitivity is reported qualitatively and may vary depending on specimen quality, bacterial load, and operator expertise

terial pathogenicity, physiology, and drug resistance [10,17]. Transmission EM (TEM) allows a detailed visualization of mycobacterial cell wall, [1,2,7,8]. TEM distinguishes the individual layers and demonstrates the thick, compact envelope that underlies acid-fastness. TEM has also elucidated polar growth in mycobacteria, showing that cell wall elongation occurs predominantly at one or both poles, with „new” poles exhibiting thinner, less organized layers. This structural heterogeneity contributes to differential antibiotic susceptibility and bacterial population variability, relevant for persistence and tolerance [10]. Beyond the cell wall, TEM reveals cytoplasmic inclusions, primarily lipid droplets, which serve as energy reserves and markers of persistent states. Antibiotic or nutrient stress increases the number and size of these inclusions, resulting in characteristic “foamy” morphologies associated with drug tolerance [13]. TEM is invaluable for studying drug effects on the cell wall; β-lactams induce peptidoglycan defects, while isoniazid disrupts mycolic acid layers, leading to vesicle formation and partial detachment of the lipid envelope [20]. Scanning EM (SEM) complements

TEM by providing high-resolution three-dimensional surface imaging, showing topography, roughness, mucoid forms, and extracellular matrix, particularly in nontuberculous mycobacteria and biofilms - structures critical for antibiotic resistance and chronic infection [4,17].

The recent advances in cryo-electron microscopy (cryo-EM) enable the visualization of cells in a near-native state without chemical fixation, preserving ultrastructural integrity and allowing a molecular-level study of transport proteins, cell wall enzymes, and secretion systems such as ESX-1 [17]. Cryo-EM is particularly powerful for investigating resistance mechanisms, comparing structures of target proteins in sensitive and resistant strains (e.g., InhA, KatG, MmpL3, DprE1), and guiding novel drugs’ development. Together, TEM, SEM, and cryo-EM provide a comprehensive understanding of mycobacterial architecture, complementing light microscopy and molecular methods by revealing the ultrastructural foundation of their biology and pathogenesis [1–3,17].

Modern optical approaches overcome the limitations

of classical structural microscopy by bridging morphological observations with functional cell biology. Confocal laser scanning microscopy, atomic force microscopy, and live-cell microscopy enable three-dimensional and dynamic investigation of mycobacterial cells, as well as the analysis of their biophysical properties [21,22]. These techniques provide critical insights into the organization of biofilms, cellular heterogeneity within populations, interactions with the immune system, and real-time adaptive responses to stress and antimicrobial agents [21,22].

In the last decade AI-based algorithms are widely applied in light, fluorescence, electron, cryo-electron, and atomic force microscopy, enabling the automated detection of acid-fast bacteria, analysis of the ultrastructure and the live-cell dynamics, and quantitative morphometric assessments.

The application of deep learning and artificial neural networks in microscopy is substantially transforming the analysis of biological images by shifting the focus from manual to automated and quantitative approaches. These technologies enable efficient image processing and enhancement, including noise reduction and high-resolution reconstruction (super-resolution), while preserving key structural features. Significant progress has also been achieved in image segmentation, where algorithms automatically recognize and distinguish cells, organelles, and microorganisms, as well as in real-time tracking of cellular behaviour [15,16,23,24].

In this context, the application of AI in TB microscopy is focused on the automated detection of AFB in sputum smears. This process is traditionally characterized by a high labour intensity and subjectivity. Contemporary approaches based on computer vision and deep learning demonstrate high diagnostic performance, particularly in resource-limited settings. Various technological solutions have been developed, including automated microscopy systems and mobile platforms integrating smartphones with conventional microscopes for real-time analysis. Object detection architectures (e.g., YOLOv7/YOLOv8) and convolutional neural networks (CNNs), such as ResNet and EfficientNet, are widely used for detection and classification [23,24].

The integration of AI into diagnostic workflows results in high accuracy (up to ~96–97%), improved detection of positive samples, and a substantial reduction in laboratory workload through automated screen-

ing, while also enhancing result standardization. Pilot studies conducted in diverse settings—including high-burden regions in sub-Saharan Africa and Asia, as well as well-equipped laboratories in Europe and North America report a sensitivity of approximately 92% and specificity of ~98% [26].

Despite these advances, important limitations remain, including the need for high-quality annotated datasets, robust quality assurance systems, algorithmic transparency, and interoperability with laboratory information systems, as well as dependence on sample quality and limited clinical validation of some solutions [13,21,22,25].

Notably, while the WHO has endorsed AI-based computer-aided detection for chest radiography (2021), no equivalent recommendation currently exists for AI-assisted smear microscopy [27]. Therefore, AI should be regarded as a complementary tool to established diagnostic methods, including Xpert MTB/RIF, rather than a replacement for expert microbiological interpretation.

CONCLUSION

Light microscopy continues to play a key role in tuberculosis diagnosis due to its accessibility, rapid turnaround time, and applicability in resource-limited settings, while fluorescence microscopy improves the sensitivity of detection. Electron microscopy provides unique high-resolution visualization of mycobacterial ultrastructure, particularly the complex architecture of the cell wall and intracellular localization.

However, each technique has inherent limitations. Conventional microscopy shows limited sensitivity in paucibacillary samples, and electron microscopy remains technically demanding and unsuitable for routine diagnostic use.

Future efforts are focused on integrating advanced imaging techniques with artificial intelligence-based tools to improve detection accuracy, standardization, and quantitative analysis in mycobacterial research.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest to declare.

REFERENCES

1. Jankute M, Cox JAG, Harrison J, Besra GS. Assembly of the Mycobacterial Cell Wall. *Annu Rev Microbiol.* 2015;69:405–423.
2. Jarlier V, Nikaido H. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiol Lett.* 1994;123(1–2):11–18.
3. Daffé M. Unraveling the structure of the mycobacterial envelope. *Microbiol Spectr.* 2015;3(6).
4. Esteban J, García-Coca M. Mycobacterium Biofilms. *Front Microbiol.* 2018;8:2651.
5. World Health Organization. Laboratory diagnosis of tuberculosis by sputum microscopy. WHO; 2021.
6. World Health Organization. Global Tuberculosis Report 2023. WHO; 2023.
7. Liu J, Barry CE. Mycolic acid structure determines the fluidity of the mycobacterial cell wall. *J Biol Chem.* 1996;271(47):29545–29551.
8. Bhamidi S, Scherman MS, Jones V, et al. Detailed structural and quantitative analysis reveals the spatial organization of the cell walls of in vivo grown Mycobacterium leprae and in vitro grown Mycobacterium tuberculosis. *J Biol Chem.* 2011;286(27):23168–23179.
9. Chiaradia L, Lefebvre C, Parra J, et al. Dissecting the mycobacterial cell envelope and defining the role of mycomembrane. *Sci Rep.* 2017;7:12807.
10. Vijay S, Hai HT, Thu DDA, et al. Ultrastructural analysis of cell envelope and accumulation of lipid inclusions in clinical Mycobacterium tuberculosis isolates. *Front Microbiol.* 2017;8:2681.
11. Eskandarian HA, et al. A role for the cell wall in mycobacterial mechanical morphotype switching. *Curr Biol.* 2017;27(24):4093–4105.
12. Clinical and Laboratory Standards Institute (CLSI). Laboratory Detection and Identification of Mycobacteria. CLSI guideline; 2018.
13. Dufrêne YF. Atomic force microscopy in cellular microbiology: from cell surfaces to single molecules. *Cell Microbiol.* 2021;23(6):e13324.
14. Marais BJ, Brittle W, Painczyk K, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. *PLoS One.* 2011;6(12):e28697
15. Melendez J, Hsieh A, Lam R, et al. Automated detection of Mycobacterium tuberculosis in Ziehl–Neelsen-stained sputum smears using convolutional neural networks. *Sci Rep.* 2016;6:27327.
16. Shanmugam ST, et al. Deep learning–based automated detection of acid-fast bacilli in digitized microscopy images. *PLoS One.* 2021;16(4):e0249227.
17. Hoffmann C, Leis A, Niederweis M, et al. Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections. *Proc Natl Acad Sci U S A.* 2008;105(10):3963–3967.
18. Tattersfield, A. (2005) Toman’s Tuberculosis: Case Detection, Treatment and Monitoring: Questions and Answers, 2nd Edition. Occupational and Environmental Medicine, 62, 70.
19. HANDBOOK FOR THE BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS. PART 1: SMEAR MICROSCOPY UPDATE / Program “Strengthening of the Network of Tuberculosis Laboratories in the Region of the Americas” -- Lima: ORAS - CONHU; 2018.
20. Wu Y, Zhou A. In situ, real-time tracking of cell wall topography and nanomechanics of antimycobacterial drugs treated Mycobacterium JLS using atomic force microscopy. *Chem Commun (Camb).* 2009;(45):7021–7023.
21. Zhang X, et al. Machine learning analysis of AFM images reveals mechanical biomarkers of mycobacterial drug tolerance. *Biophys J.* 2022;121(3):467–479.
22. Kilfoil ML, et al. AI-assisted tracking of single bacteria in live-cell microscopy. *Cell Rep Methods.* 2022;2(3):100201.
23. Chen WC, Chang CC, Lin YE. Pulmonary tuberculosis diagnosis using an intelligent microscopy scanner and image recognition model for improved acid-fast bacilli detection in smears. *Diagnostics (Basel).* 2024;14(16):1787.
24. English P, Morrison MJ, Mathison B, Enrico E, Shean R, O’Fallon B, Rupp D, Knight K, Rangel A, Gilivary J, Vance A, Hatch H, Lin L, Ng DP, Shakir SM. Use of a convolutional neural network for direct detection of acid-fast bacilli from clinical specimens. *Microbiol Spectr.* 2025 Aug 5;13(8):e0060225.
25. Fu HT, Tu HZ, Lee HS, Lin YE, Lin CW. Evaluation of an AI-Based TB AFB Smear Screening System for Laboratory Diagnosis on Routine Practice. *Sensors (Basel).* 2022 Nov 4;22(21):8497. doi: 10.3390/s22218497.
26. Mbulayi O, Djungu SJ, Aketi L, Koulali MA, Azzaoui H, Koullali R, El Mzibri M, Chaoui I, Tayalati Y. Tuberculosis diagnosis using artificial intelligence: current trends and future prospects. *Front Med (Lausanne).* 2026 Jan 7;12:1569615. doi: 10.3389/fmed.2025.1569615. PMID: 41585266; PMCID: PMC12825227
27. World Health Organization. (2021). Determining the local calibration of computer-assisted detection (CAD) thresholds and other parameters: A toolkit to support the effective use of CAD for TB screening. <https://apps.who.int/iris/handle/10665/345925>.